Ecological Traits of the Algae-Bearing Tetrahymena utriculariae (Ciliophora) from Traps of the Aquatic Carnivorous Plant Utricularia reflexa

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Ecological Traits of the Algae-Bearing *Tetrahymena utricullariae* (Ciliophora) from Traps of the Aquatic Carnivorous Plant *Utricularia reflexa*

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ABSTRACT

Trap fluid of aquatic carnivorous plants of the genus *Utricularia* hosts specific microbiomes consisting of commensal pro- and eukaryotes of largely unknown ecology. We examined the characteristics and dynamics of bacteria and the three dominant eukaryotes, i.e., the algae bearing ciliate *Tetrahymena utriculariae* (Ciliophora), a green flagellate *Euglena agilis* (Euglenophyta), and the alga *Scenedesmus alternans* (Chlorophyta), associated with the traps of *Utricularia reflexa*. Our study focused on ecological traits and life strategies of the highly abundant ciliate whose biomass by far exceeds that of other eukaryotes and bacteria independent of the trap age. The ciliate was the only bacterivore in the traps, driving rapid turnover of bacterial standing stock. However, given the large size of the ciliate and the cell11 specific uptake rates of bacteria we estimated that bacterivory alone would likely be insufficient to support its apparent rapid growth in traps. We suggest that mixotrophy based on algal symbionts contributes significantly to the diet and survival strategy of the ciliate in the extreme (anaerobic, low pH) trap-fluid environment. We propose a revised concept of major microbial interactions in the trap fluid where ciliate bacterivory plays a central role in regeneration of nutrients bound in rapidly growing bacterial biomass.

Keywords

Algae-bearing *Tetrahymena*; aquatic *Utricularia*; bacterial turnover rate; ciliate bacterivory and mixotrophy; microbial interactions; trap fluid.

INTRODUCTION

The genus *Utricularia* (Lentibulariaceae) is the most species-rich and widely distributed genus of carnivorous plants in aquatic systems (Taylor 1989). Commonly known as bladderworts, these plants have morphologically well distinguishable organs – bladder-like traps that allow the capture of planktonic organisms and other allochthonous material. The traps have received considerable attention regarding their intriguing roles in plant metabolism and nutrient acquisition, and the mechanisms of trap functioning have been well described (Adamec 2011; Alkhalaf et al. 2009; Harms 1999, Płachno et al. 2012). Besides their role in the acquisition of nutrients, the traps appear to represent a highly specific environment with distinct microbial communities (microbiomes) that colonize the trap lumen (Sirova et al. 2009, 2011).

On the one hand, the trap-associated microorganisms live under extremely nutrient rich conditions with regard to organic carbon, phosphorus, and nitrogen (Sirova et al. 2009, 2010, 2011). Table 1 characterizes the physico-chemical properties in *Utricularia* trap lumen, detailing the highly specific aquatic environment with conditions of low pH, frequent anoxia or very low concentrations of dissolved oxygen, extremely high concentrations of total and dissolved organic carbon with high proportions of simple organic carbon compounds, and relatively high concentrations of different chemical species of total and dissolved nitrogen and phosphorus. A general trend of increasing nutrient concentrations from young to old traps is evident from the previously published results (see Table 1 for details). Moreover, *Utricularia* plants exude up to 25% of their photosynthates, rich in easily biodegradable molecules such as monosaccharides and amino acids, into the trap lumen of the youngest traps (Sirova et al. 2011). In addition, decaying remains of trapped prey organisms (zooplankton, algal and cyanobacterial cells) as well as other detritus significantly enrich dissolved organic carbon (DOC) and particulate organic material pools found in the trap fluid (Borovec et al. 2012; Sirova et al. 2011). The high nutrient and DOC concentrations (Sirova et al. 2009) exceed by as much as one order of magnitude concentrations found in dystrophic water bodies (Turman 1985) where *Utricularia* typically grow.

On the other hand, the trap lumen represents a harsh environment, 1 characterized by low pH values (range 4.2-7.2, Sirova et al. 2003, 2009) and often complete anoxia (Adamec 2007; for details see also Table 1 in this paper), which can lead to the death of trapped organisms that are not adapted to survive such conditions (Płachno et al. 2012). Such extreme environmental conditions may yield specific microbial food webs, dominated by otherwise

rare and exotic taxa of protists capable of exploiting the special environment.

Utricularia trap lumen is an environment completely sealed from the outside, except for short periods (lasting only a few milliseconds) when the trap "fires" and aspirates, filling about 40% of its volume with the surrounding water. After such "firing" events, the trap seals tightly again and actively pumps "particle-free" water out of the lumen, resulting in the formation of negative pressure inside (Adamec 2011). Therefore, once a microbial cell from the surrounding environment is engulfed by the trap, it is unable to escape until the trap walls are damaged or trap senesces and ceases to perform its function. Thus, there are only two possible fates for the trapped organisms – they are either digested or, due to their ecological potential, they adapt to this specific environment and become commensals during the trap's life span.

In accordance with the latter possibility, there is increasing evidence that some specialized prokaryotes and protists form a peculiar trap microbiome (Alkhalaf et al. 2009; Płachno et al. 2012; Sirova et al. 2011; Alcaraz et al. 2016). However, very little is known about their specific interactions and their roles in plant life cycle and nutrition. In studies of the bacterial assemblages living in traps (e.g. Sirova et al. 2009, 2011, Alcaraz et al. 2016), several authors reported the occurrence of highly abundant populations of mixotrophic ciliates (e.g. Płachno et al. 2012) in numbers far exceeding those of natural surface waters. The taxonomic affiliation of these ciliates was unclear; however, it has finally been identified as a previously unknown, algae-bearing *Tetrahymena* species. This species is described as *Tetrahymena utricularіae* n. sp in our accompanying publication (Pitsch et al., this issue).

Here, we report on the food web interactions between commensal bacterial assemblages and the mixotrophic ciliate species (*T. utricularіae*) in the traps of *Utricularia reflexa,* a frequently studied plant model organism (Adamec 2007, 2011, 2014, 2015; Borovec et al. 2012; Płachno et al. 2012; Sirova et al. 2011, 2014). While the protistan biomass in traps is known to be largely dominated by the ciliate, its ecology has not been studied. Thus, the aims of our study were: (i) to characterize the extremely simplified food web structure of the indigenous microbial communities in traps of different age, (ii) to quantify bacterivory rates of the algae-bearing ciliate *Tetrahymena utricularіae* by using fluorescently labeled bacteria as prey surrogates, (iii) to examine bacterial turnover rates related to ciliate bacterivory, with the ciliate being the only bacterivore detected in traps, (iv) to estimate how mixotrophy contributes to the diet and survival strategy of the ciliate in this specific environment, and (v) to compare bacterivory rates of algae-bearing ciliates with rates determined for aposymbiotic ciliates of the same species adapted to high bacterial food concentrations.

MATERIALS AND METHODS

Experimental plant cultivation and trap fluid collection

The rootless aquatic carnivorous plant, *Utricularia reflexa* Oliv., was originally collected by D. Sirova from the Okavango Delta, Botswana in 2005. The ecological relations of *U. reflexa* microhabitats have never been studied at Okavango Swamp or elsewhere. We only know that this species grows there in relatively shaded shallow stands of taller cyperoids or graminoids (*Phragmites* sp., *Cyperus* spp.). *Utricularia reflexa* plants used in our experiments were cultivated indoors in 3-liter aquaria, with *Carex acuta* litter as a substrate (see Adamec 2007). Thus, our culture using Carex litter partly mimics the dystrophic water chemistry 1 at *U. reflexa* sites. The fact that the plant has been growing under such culture conditions for over years confirms that these conditions are close to the natural ones. The cultivation water can be classified as meso-oligotrophic and slightly humic according to the concentration of dissolved nutrients (data not shown).

The plant possesses morphologically well distinguishable 4-6 mm large traps (volume ca. 12-24 µl) that capture animal prey and host microbial communities. The trap fluid was collected from adult, approximately 25 cm long *U. reflexa* shoots using a thin glass capillary attached to a peristaltic pump (see Sirova et al. 2003). Samples of approximately 900 µl were obtained by pooling trap fluid of the same age from 10 individual plants. Shoots of these plants were divided into three segments: the apical part contained 4-5 mature nodes with young traps (referred to as "young"), followed by the mature ("mature") and old ("old") segments each containing 6-7 leaf-nodes bearing traps. Each node usually bore 2-3 traps. The collected trap fluid was processed immediately after collection. Triplicate subsamples of 200 µl of the pooled trap fluid were used for protistan grazing experiments and 300 µl of the sample were immediately preserved for further analysis of microbial components living in the fluid, as detailed below.

Quantification of microbes in trap fluid – general approach

The trap fluid is an environment with extremely high concentrations of DOC (Sirova et al. 2011), bacteria (10^7-10^8 cells ml-1) and a few prominent protistan species found in concentrations exceeding 10^4 cells ml-1. Thus, after preserving (see below for details), microbial samples were diluted 10-100 times with particle-free MQ water to achieve a suitable distribution of microbes on filter surfaces prior to their counting via epifluorescence or phase contrast microscopy.

Bacterial abundance and sizing

Samples were preserved with formaldehyde (2% final concentration) and diluted 10-20 times with particle-free MQ water. A 1-ml subsample was filtered onto black 0.2-µm pore-size filters (OSMONIC INC., Livermore, USA). Bacteria were stained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, 0.2% final concentration), and enumerated by epifluorescence microscopy (Olympus AX 70) as described previously (Šimek et al. 2008). At least 500 bacterial cells were counted per sample at 1,000 x magnification. Cell sizing (>300 cells per sample), based on measuring cell width and length parameters in DAPI-stained preparations (Posch et al. 1997), was conducted by using a semiautomatic image analysis system (NIS-Elements 3.0, Laboratory Imaging, Prague, Czech Republic). In addition to trap fluid, triplicate samples of the *U. reflexa* cultivation water were processed as outlined above.

Enumeration and sizing of protists via epifluorescence microscopy

Triplicate samples of the trap fluid (200 µl) were preserved with the Lugol-formol-thiosulfate decolorization technique (Sherr and Sherr 1993), diluted 1:10 with particle-free water, stained with DAPI, and filtered onto black 1-µm pore-size filters (OSMONIC INC.). For the quantification of the eukaryotic community, we focused on the three most prominent in terms of numbers and biomass and easily distinguishable species of protists: the ciliate *Tetrahymena utriculariae* (Pitsch et al., the accompanying paper), the euglenoid flagellate, *Euglena agilis* (Adamec and Komarek 1999; Ciugulea and Triemer 2010), and the algal species *Scenedesmus alternans*. At least 300 protistan cells were counted per sample at 400 x magnification via epifluorescence microscopy as described previously (Šimek et al. 2008). To calculate mean cell volumes (MCV) of *T. utriculariae* (ellipsoid shape), *S. alternans* (prolate spheroid shape), and *E. agilis* (half ellipsoid and cone on elliptic base, Hillebrand et al. (1999)), lengths and widths of 100 individual cells were measured manually on-screen with a built-in tool of a PC based image analysis system (NIS-Elements 3.0, 1 LIM, Prague).

Tracer technique to estimate ciliate bacterivory

Ciliate bacterivory was estimated using fluorescently labeled bacteria (FLB, Sherr and Sherr 1993) prepared from a batch bacterial culture of *Limnohabitans planktonicus* grown in nutrient rich media (Kasalicky et al. 2013) and harvested at late exponential growth phase. The growth condition yielded bacteria with the MCV of 0.39 μm^3, which corresponded with the MCV of bacteria in the traps (0.42 μm^3).

Ciliate FLB uptake rates were determined in duplicate short-term experiments as described in detail by Šimek et al. (2008). Briefly, a tracer amount of FLB, corresponding to 8% of total bacterial abundance in the traps, and 10 minutes exposure time at 20 °C resulted in well distinguishable numbers of tracer bacteria in ciliate food vacuoles (Fig. 1D-E). After exposure, samples (200 µl) were preserved with the Lugol-formol-thiosulfate decolorization technique (Sherr and Sherr 1993) and then diluted 1:10. Two hundred µl subsamples were stained with DAPI, filtered onto black 1-µm pore-size filters, and protistan uptake rates were determined via epifluorescence microscopy at 1000 x magnification as described in Šimek et al. (2000). Average uptake rates were multiplied by ciliate abundances to estimate total ciliate bacterivory rates in the fluid of traps of different age referred to as "young", "mature" and "old".

Cultivation of green and symbiont free (aposymbiotic) *Tetrahymena utricularia*e

Single traps of *U. reflexa* were separated from the plant and transferred into a small drop (300 µl) of autoclaved mineral water (Volvic). The trap wall was opened under microscopic control with two dissecting needles, thus protists inside the trap were released into the surrounding drop of water (Fig. 1A-B). Single symbiont-bearing ciliates were aspirated in an ultrafine sterilized glass pipette under a dissection microscope, to establish clonal isolates and cultures originating from several individuals. Captured ciliates were first kept in 200 µl autoclaved mineral water in 96-well plates at 18 °C, a 12 h light (10 µmol m^{-2} s^{-1})/12 h dark cycle, and under aerobic conditions. Half of the wells received 50 µl of a mixed bacterial suspension growing on wheat grains, whereas the other wells only contained autoclaved mineral water. In case of successful growth in wells, ciliate isolates were transferred into 250 ml tissue culture flasks (TPPR), filled with 50 ml of autoclaved mineral water (either enriched with bacteria or not). Cultures of symbiont bearing ciliates were maintained for several months (Pitsch et al., the accompanying paper). Nevertheless, several ciliate isolates kept under aerobic conditions with saturating bacterial food gradually lost their algal symbionts (over several weeks) – even when cultures were grown at a 12 h light/12 h dark cycle. It thus seems that the ciliates switched from the symbiotic mode towards heterotrophy, feeding exclusively on bacteria. Bacterial uptake rates of aposymbiotic ciliates were determined twice during the exponential growth in FLB direct-uptake experiments with a FLB tracer amount of approximately 5% of total bacterial abundance in these cultures (7 and 12 x 10^6 bacteria ml^{-1}) and 5 to 10 minutes exposure times.

In-vivo observations of ciliates and inspection of other *Utricularia* species

In-vivo observations were conducted with a Zeiss Axio Imager M1 at magnifications of 100 × to 1,600 × with bright field, phase, and differential interference contrast. Photographs were taken with a Canon EOS 1000. Besides analyses of trap fluid sampled from *U. reflexa*, we also made in-vivo observations of the occurrence of *T. utricullariae* in the trap lumen of the following seven *Utricularia* species: *U. australis*, *U. bremii, U. aurea*, *U. inflata*, *U. purpurea*, *U. stygia*, and *U. vulgaris.* The former two *Utricularia* species were collected from a dystrophic sand-pit pool at Branna (near Třeboň, see Adamec 2009), while the latter two species (collected in the Czech Republic) were taken from an outdoor dystrophic container mimicking natural conditions (Adamec 2007) of the collection of aquatic 1 plants at the Institute of Botany in Třeboň (Czech Republic). *Utricularia aurea* (collected in Cambodia) were grown in 3-liter indoor aquaria, while *U. inflata* (collected from New Jersey, USA) and *U. purpurea* (collected in Florida, USA) were grown in a 300 liter plastic container under dystrophic conditions in a naturally-lit greenhouse (Adamec 2007).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.04 (GraphPad Software, INC, USA). Using Kruskal-Wallis One-way ANOVA followed by Dunn's multiple comparison test, we analyzed the differences in grazing rates of (i) symbiont-bearing *T. utricullariae* living in *U. reflexa* traps of different age and of (ii) the symbiont-bearing versus the aposymbiotic ciliate populations grown in bacteria-enriched cultures. One-way ANOVA followed by Tukey post-test were used to analyze the differences in bacterial abundance and biomass in traps of *U. reflexa* of different age.

RESULTS

Microbial community in the traps of *Utricularia reflexa*

In line with the increasing trends in nutrient availability (Table 1), bacterial numbers and biomass (Fig. 2A, 2C) increased significantly (ANOVA followed by Tukey post-test, $P<0.001$) from young (47 x 10^6 bacteria ml^{-1}) to old traps (276 x 10^6 bacteria ml^{-1}), with a mean bacterial cell volume (MCV) of around 0.42 µm3. The protistan community inside young, mature, and old traps shared common features (Fig. 2A-B). It was predominantly composed of only a few morphologically easily distinguishable species: the newly described algae-bearing ciliate *Tetrahymena utricullariae* (Pitsch et al., the accompanying paper), the

green flagellate *Euglena agilis*, and the alga *Scenedesmus alternans.* These three taxa clearly dominated in numbers and biomass of eukaryotes (Fig. 2A-C), with an increasing trend towards old traps, which was best reflected by the pronounced increase of *S. alternans* (~3-times), and least apparent for ciliate numbers (factor of 1.47). Notably, we only rarely detected other eukaryotes - mainly coenobia of unidentified *Scenedesmus* spp. in old traps. Regardless of the trap age, we observed the remains of metazoan prey such as rotifers, copepods, ostracods and daphnids. DAPI-stained detrital particles and amorphous material were generally found more frequently in old traps.

The overall microbial biovolume in the traps was clearly dominated by *T. utricullariae*, accounting for 90% in young traps and 79% in old traps (Fig. 2C). Interestingly, the proportion of bacterial biovolume was much smaller, but showed an increasing trend from 3.1% in young to 14.2% in old traps.

Microbial community in the water surrounding *U. reflexa* plants

Generally, microbial communities in water surrounding the plants in the aquaria were strikingly different from those in the trap fluid. For instance, bacterial abundance was lower by 1-2 orders of magnitude, $1.66 \pm 0.04 \times 10^6$ bacteria ml^{-1} (mean ± SD), with much smaller bacterial MCV of 0.111 ± 0.012 μm^3 than in the trap fluid. Heterotrophic nanoflagellate counts were $0.419 \pm 0.027 \times 10^3$ cells ml^{-1} in the surrounding water, while this group of bacterivorous flagellates was completely absent in trap fluid regardless of its age. Ciliate counts were four orders of magnitude lower than those in the trap fluid, i.e. 4.7 ± 1.4 cells ml^{-1} (cf. Fig. 2B). Among ciliates we observed species of the class Prostomatea (*Balanion planctonicum* and *Urotricha* spp.) and, very rarely, a few individuals of the classes Spirotrichea (*Rimostrombidium* sp., *Halteria*-like species) and Oligohymenophorea (Peritrichia) (data not shown).

Interestingly, we found ciliates morphologically closely resembling the aposymbiotic *T. utricullariae* (based on cell shape and size, shape of micro- and macronucleus) in the surrounding water, i.e. the morphotypes that developed in the bacterial infusion cultures (see below), while not a single ciliate cell with algal symbionts (typical for trap fluid), was observed. However, about 20% of ciliates (approximately 1 cell ml^{-1}) in the surrounding water were obviously resting cysts without symbionts (for the life cycle of the ciliate see Pitsch et al., the accompanying paper) observed also in late stages of the bacteria-fed clonal ciliate batch cultures.

Algal symbionts of *Tetrahymena utricullariae*

The most striking feature of *T. utricullariae* is the symbiosis with the alga *Micractinium* sp. of the class Chlorophyta (Fig. 1C-D). For a detailed description and taxonomic affiliation of algal symbionts we refer to Pitsch et al. (this issue). Ciliates were packed with algae, and the number of symbionts was significantly positively related to the size of their hosts (Fig. 3). A ciliate cell harbored on average 52 symbiotic algae and we never observed aposymbiotic, i.e., symbiont-free specimens in the trap fluid.

Trophic mode of ciliates in traps - bacterivory versus mixotrophy

The FLB tracer technique showed that the only phagotrophic protist ingesting bacteria in traps was *T. utricullariae*. Green ciliates living in traps took up, on average, 5-7 FLB per individual during the 10 min exposure time (median, 6-9 FLB per ciliate in different aged traps, see an example in Fig. 1D). This corresponds to ingestion rates of 263-342 bacteria ciliate^{-1} h^{-1} (Fig. 2D). Notably, the dramatic increase in bacterial numbers in old traps (Fig. 2A) was not reflected correspondingly in the ingestion rates of ciliates that were only slightly, but insignificantly, higher in old compared to young and mature traps (Kruskal-Wallis One-way ANOVA followed by Dunn's multiple comparison test, $P>0.05$). Bulk bacterivory rates of the abundant ciliates (Fig. 2B), however, indicated a very rapid bacterial standing stock turnover time in fluid of young (5.0 d-1) and mature traps (4.4 d^{-1}), compared to much slower turnover in old traps (1.5 d^{-1}, cf. Fig. 2A, 2D). We also estimated the role of carbon gained through bacterivory for the ciliates' energetic balance. Taking into account the ciliate MCV (14,150 µm^3), its bacterivory rates (Fig. 2D), the MCV of bacterial prey (0.42 µm^3), and assuming a 40% gross growth efficiency and bacterivory as the only carbon resource, the symbiont bearing ciliate populations (Fig. 2B) in traps would have generation times of 10.3-13.4 days.

Symbiont-bearing ciliates were isolated from plant traps and grown in monoxenic batch cultures. Subsets of these cultures were then transferred onto an exclusive diet of bacteria grown on a wheat infusion, which led to a gradual loss of symbionts, i.e., ciliates became aposymbiotic and grew as heterotrophs (Fig. 1E, 4A). This transformation permitted comparison of growth parameters and bacterial ingestion rates of symbiont-bearing versus aposymbiotic ciliate populations. Aposymbiotic ciliates grown on bacteria showed 3-fold higher ingestion rates (912 and 1021 bacteria ciliate^{-1} h^{-1}) compared to green individuals in the traps of different age (Fig. 2D). The ingestion rates were significantly different (Kruskal-Wallis One-way ANOVA followed by Dunn's multiple comparison test, $P<0.05$). Using the same assumptions of conversion efficiencies to estimate growth rates of aposymbiotic ciliates,

we estimated that they could double within 3.3 - 3.9 days. These growth estimates are slightly higher than those calculated from growth curves (i.e. based on the cell number increase) of aposymbiotic ciliates fed with bacteria (Fig. 4A).

We also tested conditions under which aposymbiotic, bacteria-fed ciliates re-acquired their symbionts. Bacteria previously grown in wheat infusion supported relatively stable ciliate growth, with one doubling approximately every 6 days (Fig. 4A). The light-grown populations exposed to combinations of bacterial food and added endosymbiotic algae grew relatively fast during the first three days (Fig. 4B). Thereafter, numbers leveled 1 off or slightly decreased in parallel with an increasing proportion of ciliates with re-acquired symbionts. After 44 days of co-cultivation, approximately 80% of ciliates had reestablished symbiosis with the algae. The cultures amended with symbiotic algae alone showed only a slow decrease in ciliate numbers and a surprisingly low rate of symbiont acquisition (Fig. 4C). Nevertheless, almost 50% of ciliates harbored algae after 44 days of incubation.

Unique appearance of the mixotrophic *T. utricullariae* in traps of *U. reflexa*

We inspected the fluid from the traps of different age in seven other *Utricularia* species (see Materials and Methods), but could not detect this ciliate in any plant other than *U. reflexa*. Similarly, by combining in vivo observations and by the inspection of fixed material from the surrounding medium of different *Utricularia* species, we did not observe cells of this ciliate species outside of the traps. In addition, we conducted a three month co-cultivation experiment combining *U. reflexa* (two different populations from Botswana and Zambia) together with *U. aurea* and *U. stygia* in one 20 liter outdoor aquarium. With this experimental approach, we aimed to study a possible cross-infection of *U. aurea* and *U. stygia* traps with green ciliates originating from traps of *U. reflexa*. However, even after three months of co17 cultivation, the green *Tetrahymena* could only be detected in the traps of both *U. reflexa* populations, but not in the two other plant species.

DISCUSSION

Key players in the *U. reflexa* trap fluid

In many ways the trap-associated microbial communities in *Utricularia* are similar to food webs described from the phytotelmata of other carnivorous plant species, especially those found in the pitchers of terrestrial genera such as *Sarracenia* and *Nepenthes* spp. (for review see Kitching 2000). All of these communities are aquatic and occur within larger ecosystems

(usually terrestrial) as a series of units scattered spatially. A complete food web exists within each of these isolated habitats and many species are endemic to these special environments. The communities of metazoan animals or top predators are relatively simple, ranging from one or two to up to twenty or thirty. In many of these environments, especially pitchers, the conditions can be extreme, for example with regards to pH (Adlassnig et al. 2011). Hence, both bottom-up and top-down control mechanisms shape these environments. There is a strong succession and structuring of the food webs as they occur within containers that are themselves relatively short-lived and the plant and microbial life cycles are thus closely coupled. They represent replicated, faunistically and structurally relatively simple habitats which provide excellent model study systems in ecology (Srivastava et al. 2004). The traps of carnivorous *Utricularia* represent some of the most miniaturized of these habitat types. They differ in their virtually complete isolation from the surrounding environment, very low oxygen concentrations likely lethal to most metazoans which occur and even breed in other phytotelmata, a high degree of selectivity and very rapid aging and turnover (Adamec 2007, 2011). *Utricularia* traps are also unique in the sheer numbers of ciliates present. The only environments where comparable numbers of ciliate protozoa can be found are the animal rumen and activated sludge, where protozoan abundances in the order of 8-9 x 10^5 ml^{-1} and 4 x 10^4 ml^{-1}, respectively, have been reported (for example Abraham et al. 1997; Duarte Messana et al. 2012). However, the dense communities represent total populations of many different species.

Our study describes the presence of this extremely simplified, but abundant eukaryotic microbial community (compare Płachno et al. 2012; Sirova et al. 2009) hosted in the traps of the carnivorous plant *U. reflexa* (Fig. 5). Likely due to the harsh physico-chemical environment in the trap lumen (Table 1 and references therein), the commensal 1 mixotrophic ciliates were clearly the dominating components of the biomass of the trap communities, far exceeding even that of bacteria (Fig. 2C). The mixotrophic ciliate in *U. reflexa* was misclassified as *Paramecium bursaria* in an earlier study (Płachno et al. 2012), and bacteria were proposed to be the major carbon source for that ciliate. However, detailed morphological analyses in concert with sequencing data clearly showed that this ciliate species is affiliated with the genus *Tetrahymena,* and it is currently described as the new species *T. utricullariae* (Pitsch et al., the accompanying paper). Notably, this is the first described mixotrophic member of this well-known ciliate genus. In addition, it seems to be an obligate commensal of *U. reflexa* traps, unable to inhabit traps of any other inspected *Utricularia* species.

Interestingly, while small heterotrophic flagellates are the main bacterivores in most

391 aquatic systems (Berninger et al. 1991; Jurgens and Matz 1992; Montagnes et al. 2008), the
392 ciliate *T. utricullariae* was the only bacterivore observed in the trap fluid. We did not observe
393 phagotrophy in the other abundant, chloroplast-bearing protist growing in the traps – the
394 euglenoid *E. agilis*, which also seemed to commonly thrive in this environment (Alkhalaf et
395 al. 2009). Thus, as in the case for the green alga *S. alternans,* photosynthesis likely could be a
396 partial source of organic carbon for their growth. We cannot exclude some role of
397 osmotrophy, namely in the diet of *E. agilis* and *T. utricullariae* under extremely high DOC
398 concentrations present in trap fluid (Sirova et al. 2009, 2011). The high and increasing
399 densities of both the euglenoid and *S. alternans* (Fig. 2A-B) indicate that these microbes
400 likely actively grow in the traps as we did not observe these morphotypes in the surrounding
401 water, except for a few unidentified individuals of the genus *Scenedesmus* (data not shown).
402
403 **The core microbial interactions in the trap fluid of *U. reflexa***
404 Based on new findings reported in this study and literature data (Table 1), we propose a
405 schematic depiction of the core microbial interactions in the trap fluid of *U. reflexa* (Fig. 5).
406 The photosynthetic activity of the plant fuels active transport of photosynthates into trap fluid
407 (Sirova et al. 2009) in parallel with production of phosphatases mainly in the young traps,
408 allowing release of organically bound phosphorus from captured prey and organic detritus.
409 The trap fluid is continuously enriched in organic molecules, such as monosaccharides,
410 alcohols, and amino acids (Borovec et al. 2012; Sirova et al. 2009, 2011) in the form of plant
411 exudates (Fig. 5). This process seems to be quantitatively more important in young traps and
412 it can significantly accelerate microbial colonization also in prey-free traps (Adamec 2011;
413 Sirova et al. 2009). The pool of exudates of plant origin can further be enriched by exudates
414 produced by the large biomass of chloroplast- or symbiont-bearing eukaryotes actively
415 growing in the trap fluid (Fig. 5). The high inorganic and organic nutrient availability fuels the
416 growth of prokaryotes, which also act as significant producers of phosphatases and of other
417 exoenzymatic activities (Sirova et al. 2009, 2011). Thus bacteria are likely responsible for
418 rapid nutrient recycling in the trap fluid.
419 Our proposed scheme attributes a central role in the trap microbiome dynamics to the
420 bulk bacterivory of mixotrophic ciliates, responsible for the rapid consummation of bacterial
421 biomass and thus recycling limiting nutrients that would otherwise be sequestered in this
422 biomass (Sherr and Sherr 1988; Sterner and Elser 2002). Imbalance in bacterial growth and
423 loss rates can represent a bottleneck in microbial nutrient regeneration and the overall nutrient
424 acquisition by the plant. However, the cell-specific phagotrophy of the green ciliate is

relatively low regarding its large cell size (Pitsch et al., the accompanying paper). Thus, ciliate phototrophy (via acquired symbionts), osmotrophy and even histophagy (Fig. 5) should be taken into account, i.e., several trophic modes are probably co-acting in the diet of the ciliates. Our study is the first one reporting this unusual trophic structure with highly specific microbial interactions and give some hints as to carbon and nutrient flows in this unique environment 1 (Fig. 5).

The intriguing role of the symbiotic alga in the ciliate lifestyle

We used the term symbiont for the green alga *Micractinium* sp. (Pitsch et al., the accompanying paper) acquired by the trap-associated ciliate even though this term encompasses a wide spectrum of possible interactions spanning from parasitism to mutualism (Paracer and Ahmadjian 2000). We managed to isolate and separately culture both symbiont bearing and aposymbiotic *T. utricularіae,* to conduct transient re-infection and grazing experiments (Fig. 1D-E, 4). Our experiments have brought insights into the potential role of the acquired alga for the nutrition and lifestyle of the ciliate. We found a flexible life strategy of the ciliate important for the species transfer through a non-commensal phase in the surrounding aquatic environment that facilitates the occupation of newly formed traps. The ability to switch between the two trophic modes of nutrition seems to be a common phenomenon also in other mixotrophic ciliate groups (Dolan 1992; Mitra et al. 2016). Symbiont infection has also been studied with the related ciliate *T. thermophila*, generally considered a symbiont-free species, in long-term co-culture experiments (Germond et al. 2013).

In *T. utricularіae*, our results suggest that symbionts are not mandatory for the survival outside of the specific conditions present in the trap lumen, i.e. when cultured aerobically with an excess of bacterial food (Fig. 4A). However, the rates of bacterivory of the symbiont-bearing ciliate were far too low to support the assumed high growth rates of ciliates in traps based on the following considerations: (i) Typical ciliate densities detected in traps (Fig. 2B) correspond to approximately 350-900 ciliates per trap of 12-24 µl volume. (ii) There is a rather limited amount of ciliate inoculum in the water surrounding the plants (only ~ 1 resting cyst ml-1). (iii) Vital traps do not release their particulate contents with engulfed cells and perform approximately 3-4 “feeding events” per day. (iv) Each trap is able to suck in ~40% of its volume during the feeding event, thus significantly diluting the inner trap fluid (Sirova et al. 2009). (v) The estimated lifespan of the traps is around 30 days (Adamec 2011, 2015). Thus, one must assume very rapid germination of the ciliate resting cysts and a high growth rate after being aspirated from the surrounding water to successfully colonize the trap

environment. Apparent rapid population growth requires an additional carbon source in addition to ciliate bacterivory (Fig. 2D). Note that even significantly higher bacterial uptake rates of the aposymbiotic ciliate population would not allow, in terms of energetic demands, more than one division per 3-6 days. The most likely explanation for the high abundance of ciliates in traps is therefore the presence of algal symbionts (around 52 per cell, Fig. 3) that provide an additional carbon source and, moreover, produce a sufficient amount of oxygen for the ciliates growing in DOC-rich environment under sub- to hypoxic conditions (Table 1; Adamec 2007).

Acquiring symbionts by the ciliates thus can have multiple benefits, although our conclusions are partly speculative since we cannot directly quantify the proportion of organic carbon provided by symbiotic alga or ciliate osmotrophy. We have to admit that due to numerous methodical constraints related to the tiny volume of the traps, prevailing anoxic conditions in the trap fluid, and co-occurrence of other abundant chloroplasts-bearing eukaryotic microbes (Fig.1, 2), the direct in situ measurement of the ciliate photosynthesis is currently impossible. However, as the walls of young traps are nearly transparent (Fig. 1A), their effect on limiting the photosynthetic activity of the symbionts is likely low, while this does not apply for older brownish and much less transparent traps; probably, the proportion of phototrophy is decreasing with the trap age.

In this context it is worth noting that symbiont-bearing ciliate populations start to gradually lose their symbionts when cultivated outside of the traps in bacterized cultures under oxygen saturating conditions. The loss of symbionts was even accelerated 1 by bacterial additions (unpubl. data). On the other hand, bacterial additions to the aposymbiotic ciliates significantly stimulated the re-acquisition of symbionts (Fig. 4B). This observation might indicate a positive effect of phosphorus acquisition through grazing on phosphorus-rich bacterial cells (Eccleston-Parry and Leadbeater 1995; Jurgens and Gude 1990; Sterner and Elser 2002). Additionally, cultures of symbiont bearing ciliates could be maintained for several months in an inorganic medium at a 12 h light/12 h dark cycle. This again points to the important contribution of mixotrophy to the diet of the ciliate, serving obviously as sort of "an on-board green garden" for organic carbon and oxygen productions in the specific trap environment.

Aposymbiotic *T. utriculariae* had significantly higher bacterial uptake rates compared to symbiont bearing ones. However, when we consider MCV of the ciliates and their uptake and division rates under bacteria saturating conditions (Fig. 4A), the rates observed would again be too low to sustain this ciliate under severe competition and reduced food resources in

493 the pelagic environment. For instance, much smaller typical planktonic bacterivorous or
494 omnivorous ciliates, exploiting bacterial prey availability approximately one order of
495 magnitude lower, in general have higher bacterial uptake rates (Šimek et al. 1996, 2000).
496 Thus our data indicates that bacterivory itself would not allow for the high ciliate biovolume
497 found in traps' fluid as no larger particulate food items were observed in ciliate food vacuoles
498 (Fig. 1C-D).
499 We cannot exclude a certain role of osmotrophy in the nutrition of *T. utriculariae* in
500 the DOC-rich environment with ample organic substrates supplied by the plant. Notably, the
501 other key protists in the trap fluid, the non-phagotrophic green euglenoids, are also known as
502 osmotrophs living in organically rich environments (Brodie and Lewis 2007). This is in line
503 with our observations that *E. agilis* increased its abundance with the trap age in parallel with
504 increasing DOC concentrations, a general trend reported by Sirova et al. (2011). It is perhaps
505 worth noting that other species of *Tetrahymena* are capable of osmotrophy as they can be
506 grown in axenic cultures (Arregui et al. 2007; Curds and Cockburn 1968). If osmotrophy can
507 meet a significant part of carbon requirements of these two protists, then the presence of intact
508 chloroplasts (the euglenoids) or symbionts (the ciliate) can also indicate their key role in
509 oxygen production as a prerequisite for the rapid growth in DOC-rich but oxygen-depleted
510 trap fluid. Moreover, *T. utriculariae* could also be partially histophagous as they are often
511 observed aggregated near animal remains (Płachno et al. 2012), and in our study even inside
512 remains of prey such as ostracods, daphnids and rotifers.
513 The algal symbionts apparently profit from being protected in ciliate cytoplasm from
514 the harsh environment in trap fluid (Table 1), while being sufficiently supplied with CO2 and
515 limiting nutrients, e.g. by phosphorus via ciliate bacterivory. Although this type of symbiotic
516 relationship under almost complete anoxia is rather unique, it generally fits the recently
517 proposed category of "a non-constitutive mixotroph" (Mitra et al. 2016) that acquires its
518 phototrophic capacity by ingesting specific algal prey. While the symbiont is cultivable
519 separately in axenic culture (Pitsch et al., the accompanying paper), after being acquired it
520 likely divides well in the ciliate as we only rarely observed similar round-shaped autotrophs
521 (3-4.5 μm diameter) in the trap fluid. The whole population of the ciliate living in traps is
522 symbiont-bearing, which indicates a transfer of symbionts during cell division from the
523 mother cell to the new generation of daughter cells (Pitsch et al., the accompanying paper)
524 and even to the cysts found in the trap fluid. However, the way how the apparently vital
525 ciliates with their intact chloroplasts and active uptake of bacteria protect themselves from
526 being killed and digested in the extreme environment in traps is beyond the scope of this

study.

Microbial populations surrounding the plant, ciliate transfer to newly formed traps

Our results demonstrated striking differences in microbial assemblages present 1 in and outside of *U. reflexa* traps. The water surrounding the plants in cultivation aquaria hosted microbial assemblages that are, in terms of densities of prokaryotes, flagellates and ciliates, typical for mesotrophic lakes during clearwater phase (cf. Posch et al. 2015; Šimek et al. 2008, 2014; Sommer et al. 2012). While small heterotrophic flagellates were present in the surrounding water, they were entirely absent in the bacteria-rich trap fluid, which again points to the extreme character of this environment. This taxonomically diverse group of bacterivores is omnipresent in water columns and sediments of the vast majority of aquatic environments of sufficient bacterial densities regardless of their oxygen status (Boenigk and Arndt 2002; Sherr and Sherr 2002). To our knowledge, there is no other nutrient-rich aquatic environment similar to the trap fluid of *U. reflexa*, where bacterial grazing control appears to depend on one mixotrophic ciliate species.

We have proposed the synergy of bacterivory, autotrophy, and perhaps osmotrophy as the major energy sources for *T. utricullariae* (Fig. 5). Generally, adverse environmental conditions induce formation of ciliate resting cysts (for the full life cycle of the ciliate see Pitsch et al., accompanying manuscript). Cyst formation started in food-depleted late stages of the symbiont bearing ciliate batch cultures and cysts were also more abundant in old traps, which might be related to the reduced trap wall transparency constraining phototrophy of the symbiont. The cysts in the ambient water (approximately 1 cell ml^{-1}), are likely fundamental for the ciliate survival strategy when released from decaying traps to the ambient environment with limited bacterial abundance and reduced DOC amounts compared to trap fluid. This is in line with our finding of only a few individuals of aposymbiotic *T. utricullariae* in the surrounding water. Notably, in natural sites or also in the dense stands within *U. reflexa* culture, very young traps without ciliates occur in the close vicinity to very old, disintegrating traps from which a high number of ciliates is released. Thus they could be effectively aspirated into young traps also by frequent firings.

Unfortunately, we currently cannot make any conclusions about the source environment of *T. utricullariae*. Since the trap content of *U. reflexa* newly collected at the Botswana location was not evaluated at that time, we cannot say whether plants were infected at the original location or during subsequent continuous cultivation in the Czech Republic. It is more likely, however, due to the fact that *T. utricullariae* has not been described previously

561 from any European sites, that the source of the inoculum is the Okawango swamp.
562
563 ## Conclusions
564 The composition of the eukaryotic communities in traps of the (sub)tropical species *U. reflexa*
565 seems to be simpler and markedly different from other species of *Utricularia* from the
566 temperate zone, hosting a rather diverse eukaryotic community (Alkhalaf et al. 2011). The
567 traps of *U. reflexa* were dominated by only a few prominent and relatively large species,
568 which are commensals clearly capable of growth in the trap fluid as population densities
569 increase with trap age. Thus, our study clearly shows that the ciliate *T. utricullariae* is not
570 simply an occasional prey item captured during feeding events of traps. Unfortunately, we do
571 not know the specific adaptations of the three eukaryotic species allowing them to
572 successfully cope with the extreme physico-chemical conditions in trap fluid (Table 1) that
573 otherwise lead to death and digestion of captured prey (Alkhalaf et al. 2011; Płachno et al.
574 2012). Our data is in a good agreement with the study by Płachno et al. (2012) on the
575 abundant eukaryotic commensals in traps of *U. reflexa*, but we did not detect a diverse
576 community of algal species in traps. However, we used also a different approach compared to
577 Płachno et al. (2012), who quantified numbers of organisms per trap and thus the large
578 variability in size and other traps' characteristics was correspondingly reflected in very high
579 standard deviations for each organismal group. In contrast, we pooled contents of many traps
580 of a comparable age for a quantification of the prominent microbes that 1 were expressed as
581 abundances and biovolumes per ml of trap fluid. We noted that all other algal species were at
582 least 1-3 orders of magnitude less abundant than the here described three eukaryotic taxa (Fig.
583 2) of large MCV. Thus the contribution of other eukaryotic taxa (not shown in Fig. 2) to the
584 overall eukaryotic biovolume is likely negligible.
585 Our results corroborate earlier findings that the microbiome of trap fluid of
586 *Utricularia* species is dominated by chloroplast or symbiont bearing eukaryotes that might
587 significantly contribute to primary production, DOC and nutrient supply to the plant (Alkhalaf
588 et al. 2011; Borovec et al. 2012; Peroutka et al. 2008; Płachno et al. 2012; Sirova et al. 2011).
589 Moreover, we propose a conceptual framework with the newly described mixotrophic ciliate
590 *T. utricullariae* acting as a key species in the trap fluid of *U. reflexa* (Fig. 5). These ciliates are
591 of high ecological relevance as they are the only bacterivores in this environment, and they
592 possess a specific life strategy based on co-acting of several trophic modes. Symbiont-bearing
593 *T. utricullariae* were only present in two populations of African *U. reflexa,* and they were not
594 detected in traps of other *Utricularia* species from other continents, nor in any other aquatic

environment. The ecological reasons for this specificity are still unknown.

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FIGURE LEDENDS

Figure 1 A-E. Microphotographs of: **A.** View of the two-layered epithelium of *Utricularia* traps. Silhouettes of green protists (euglenoid flagellates and ciliates) are already visible. **B.** After a trap was opened with a dissection-needle, protists were released. **C.** A live ciliate cell of *Tetrahymena utricullariae* (differential interference contrast) with numerous symbiotic algae. **D.** A DAPI-stained fixed symbiont-bearing ciliate. Note the red autofluorescence of algae. **E.** A DAPI-stained fixed aposymbiotic ciliate with one macro- and one micronucleus. Ingested fluorescently labeled bacteria appear as blue (D) or yellow (E) rod-shaped objects in food vacuoles, pointed out with arrows (D). Scale bar: 100 μm (A–B), 10 μm (C–E).

Figure 2 A-D. Microbial parameters in young, mature and old traps of *Utricularia reflexa*. (A-D). **A.** Abundance of bacteria and *Scenedesmus alternans*. **B.** Abundance of *Tetrahymena utricullariae* and euglenoid - *Euglena agilis*. **C.** Biovolumes of bacteria, *S. alternans, T. utricullariae,* and *E. agilis*. **D.** Bacterial standing stock turnover time (bacterial abundance related to the bulk bacterivory of ciliates) and cell-specific bacterial ingestion rate of the ciliates. Error bars (if presented) show range of values of duplicate measurements.

Figure 3. Significant relationship between ciliate cell lengths and number of algal symbionts per individual (linear regression and 95% confidence intervals, n = 40, $r^2 = 0.71$).

Figure 4 A-C. Comparison of the growth of aposymbiotic ciliates in re-infection experiments when fed by: **A.** Bacterial infusion only. **B.** Bacterial infusion in combination with the addition of isolated algal symbionts. **C.** No bacterial food and isolated algal symbionts only. Columns fills indicate the proportion of ciliates which re-acquired the symbionts (categorized in ciliates half or totally filled with algae). All treatments were under 12h light/12 dark cycle. Vertical error bars show deviations of duplicate treatments.

Figure 5. A scheme depicting potential interactions between the mixotrophic ciliate *Tetrahymena utricullariae* and other microbes, by their enzymatic capabilities, and physico chemical properties of the specific environment in trap fluid. The purple arrows indicate the major interactions and nutrient and organic carbon fluxes in the trap fluid. The black arrows indicate places where phosphatases and organic compounds are released. Compiled from published literature and data gained in this study (for details see the text).

774 Table 1. Physico-chemical properties of the environment inside *Utricularia* trap lumen, compiled from
775 published literature.

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Properties of the *Utricularia* trap environment	
pH	Species specific, in the range of 4.2-7.2, usually 5.1 (Sirová et al. 2003, 2009)
Dissolved oxygen [μM]	0.0–4.7 (Adamec 2007)
Redox potential [mV]	-24 to -105 (Adamec 2007)
Total organic carbon [mg L^{-1}]	Trap age dependent, from 400 in youngest to 1600 in older traps (Sirová et al. 2009)
Dissolved organic carbon [mg L^{-1}]	Trap age dependent, from 60 in youngest to 300 in older traps (Sirová et al. 2009)
Dissolved organic carbon composition	Includes sugars, amino acids, organic acids, and sugar alcohols (Sirová et al. 2011, Borovec et al. 2012)
Total nitrogen [mg L^{-1}]	Trap age dependent, from 20 in youngest to 80 in older traps (Sirová et al. 2009)
Dissolved nitrogen [mg L^{-1}]	Trap age dependent, from 7 in youngest to 25 in older traps (Sirová et al. 2009)
NH_4-N [mg L^{-1}]	2.0–4.3 (Sirová et al. 2014)
Total phosphorus [mg L^{-1}]	Trap age dependent, from 0.9 in youngest to 4.2 in older traps (Sirová et al. 2009)
Dissolved phosphorus [mg L^{-1}]	Trap age dependent, from 0.2 in youngest to 0.6 in older traps (Sirová et al. 2009)

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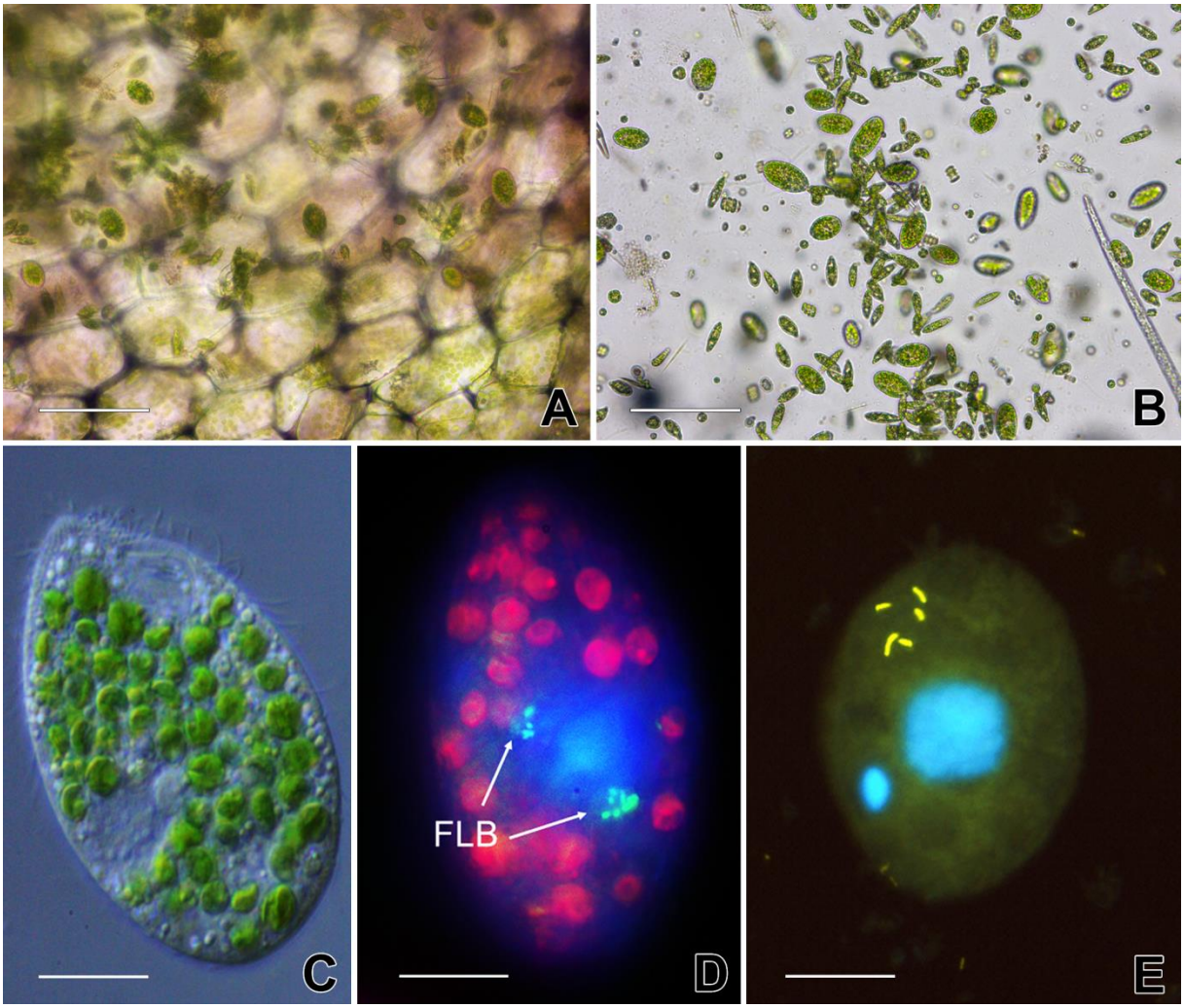
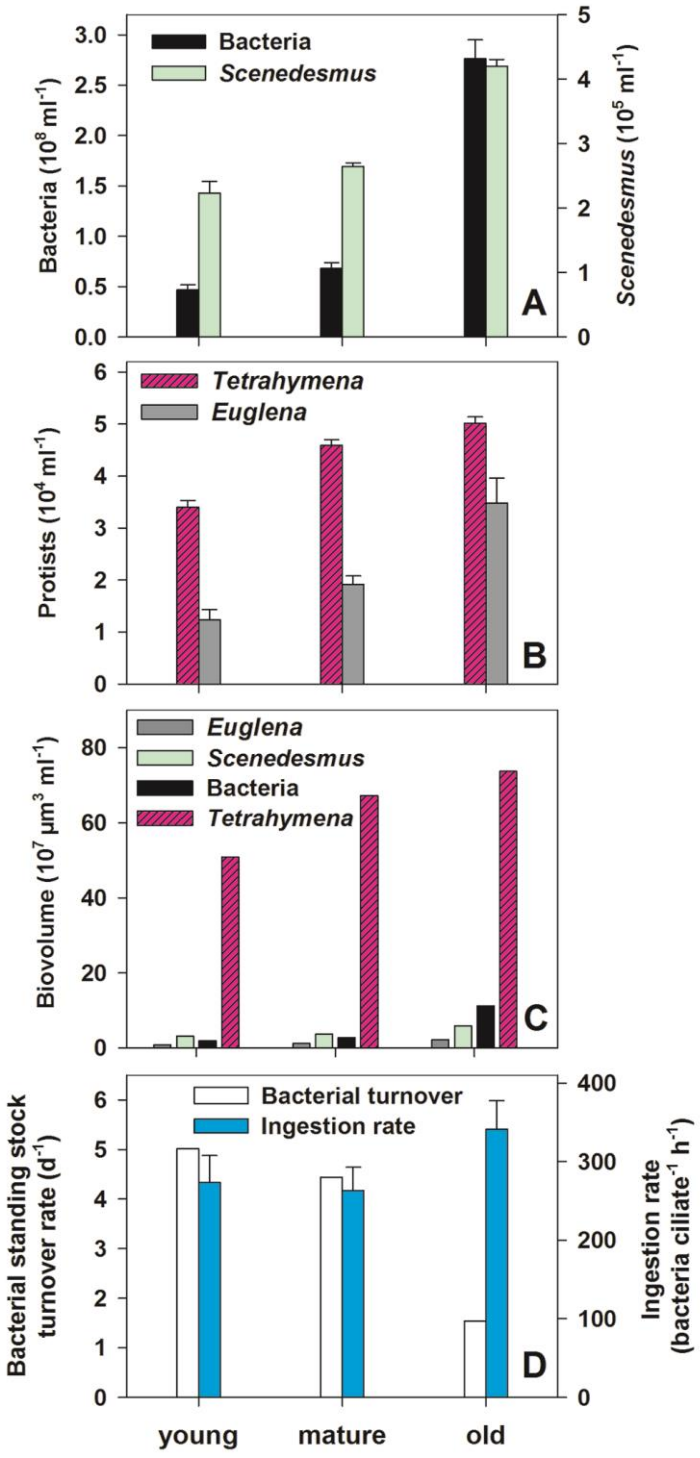


Figure 1



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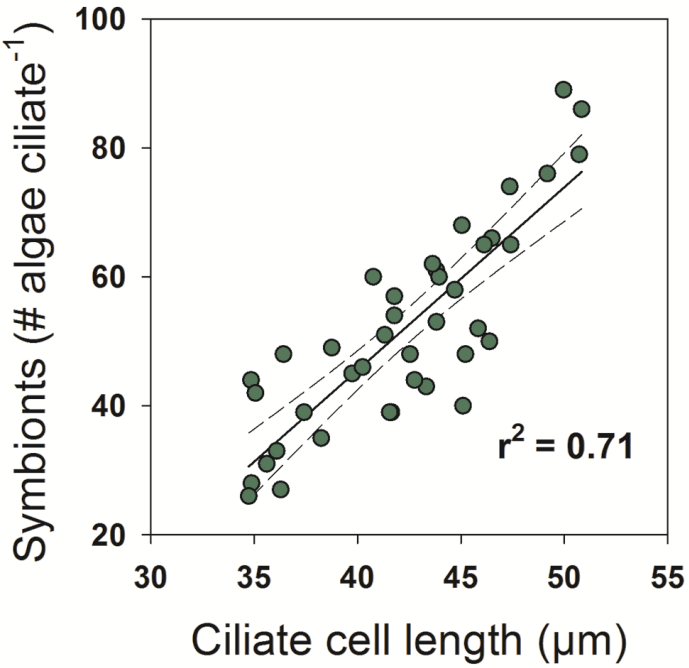
785 Figure 2



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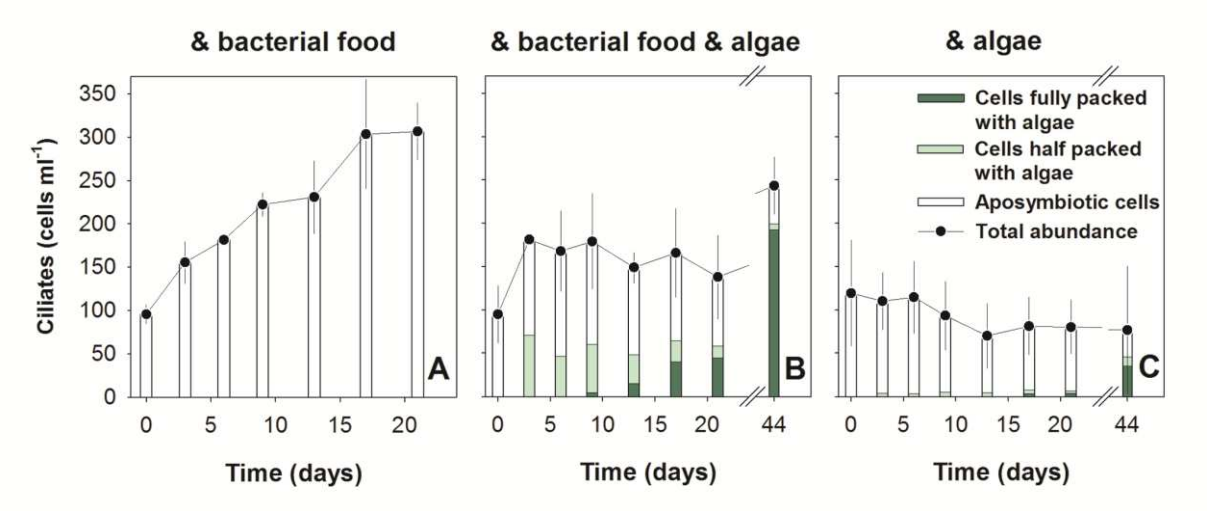
788 Figure 3



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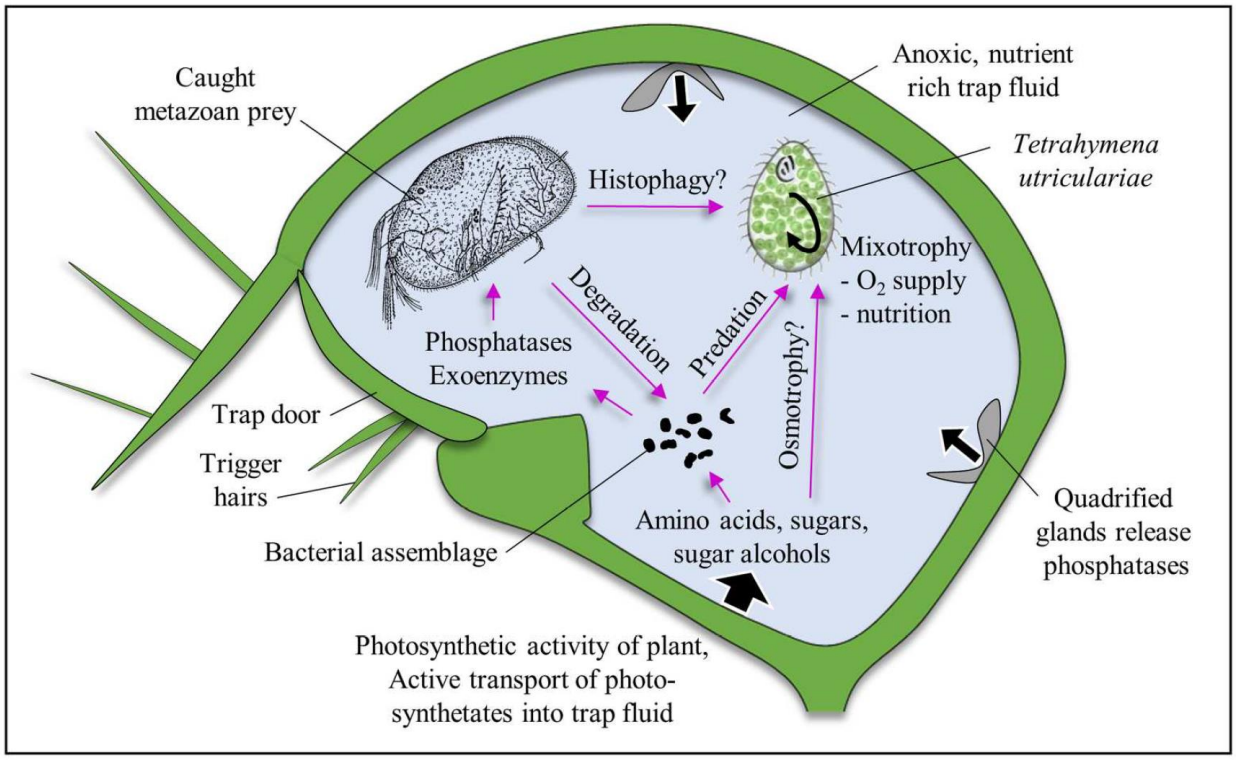
791 Figure 4



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795 Figure 5